NATIONAL TOXICOLOGY PROGRAM
Technical Report Series
No. 390

TOXICOLOGY AND CARCINOGENESIS STUDIES OF 3,3′-DIMETHYLBENZIDINE DIHYDROCHLORIDE

(CAS NO. 612-82-8)

IN F344/N RATS

(DRINKING WATER STUDIES)

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

FORWORD

The National Toxicology Program (NTP) is made up of four charter agencies of the U.S. Department of Health and Human Services (DHHS): the National Cancer Institute (NCI), National Institutes of Health; the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health; the National Center for Toxicological Research (NCTR), Food and Drug Administration; and the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control. In July 1981, the Carcinogenesis Bioassay Testing Program, NCI, was transferred to the NIEHS. The NTP coordinates the relevant programs, staff, and resources from these Public Health Service agencies relating to basic and applied research and to biological assay development and validation.

The NTP develops, evaluates, and disseminates scientific information about potentially toxic and hazardous chemicals. This knowledge is used for protecting the health of the American people and for the primary prevention of disease.

The studies described in this Technical Report were performed under the direction of the NIEHS and were conducted in compliance with NTP chemical health and safety requirements and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals. The prechronic and chronic studies were conducted in compliance with FDA Good Laboratory Practice Regulations and all aspects of the chronic studies were subjected to retrospective quality assurance audits before being presented for public review.

These studies are designed and conducted to characterize and evaluate the toxicologic potential, including carcinogenic activity, of selected chemicals in laboratory animals (usually two species, rats and mice). Chemicals selected for NTP toxicology and carcinogenesis studies are chosen primarily on the bases of human exposure, level of production, and chemical structure. Selection per se is not an indicator of a chemical's carcinogenic potential.

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NTP TECHNICAL REPORT

ON THE

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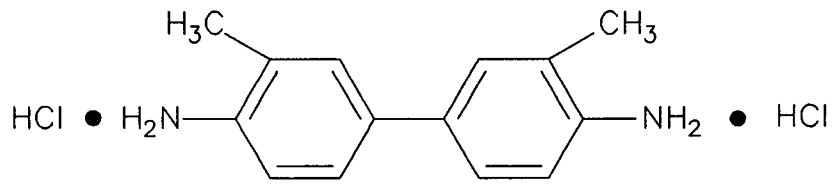
ABSTRACT



3,3′-DIMETHYLBENZIDINE DIHYDROCHLORIDE

CAS No. 612-82-8

$C_{14}H_{16}N_2 \cdot 2HCl$ Molecular Weight 285.2

Synonyms: *o*-Tolidine dihydrochloride; 3,3′-Dimethylbiphenyl-4,4′-diamine dihydrochloride; 3,3′-Dimethylbiphenyl-4,4′-biphenyldiamine dihydrochloride; 4,4′-Diamino-3,3′-dimethylbiphenyl dihydrochloride

3,3′-Dimethylbenzidine dihydrochloride is one of five chemicals being evaluated in 2-year carcinogenicity and toxicity studies as part of the NTP's Benzidine Dye Initiative. This Initiative was designed to evaluate representative benzidine congeners, benzidine congener-derived dyes, and benzidine-derived dyes. 3,3′-Dimethylbenzidine dihydrochloride was nominated for study because of the potential for human exposure during production of bisazobiphenyl dyes and because benzidine, a structurally related chemical, is a known human carcinogen.

Toxicology and carcinogenesis studies were conducted by administering 3,3′-dimethylbenzidine dihydrochloride (approximately 99% pure) in drinking water to groups of F344/N rats of each sex for 14 days, 13 weeks, or 9 or 14 months. The 14-month exposures were planned as 24-month exposures but were terminated early because of rapidly declining animal survival, due primarily to neoplasia. These studies were performed only in rats because similar studies were being performed in mice at the National Center for Toxicological Research (NCTR). Hematologic and serum chemical analyses and thyroid hormone determinations were conducted in conjunction with the 13-week and 9-month studies. Genetic toxicology studies were conducted in *Salmonella typhimurium*, Chinese hamster ovary (CHO) cells, and *Drosophila melanogaster*.

14-Day Studies: Rats were exposed to 3,3′-dimethylbenzidine dihydrochloride in drinking water at doses ranging from 600 to 7,500 ppm. All five males and one female in the 7,500 ppm group and 1/5 males in the 5,000 ppm group died. Final mean body weights were decreased in males receiving 1,250 ppm or more and in all exposed females, and final mean body weights of animals receiving 2,500 ppm or more were lower than initial weights. Water consumption decreased with increasing

chemical concentration. Compound-related effects observed in rats receiving 5,000 ppm or more included minimal to slight hepatocellular necrosis, accumulation of brown pigment (presumably bile) in individual hepatocytes, increased severity of nephropathy relative to controls, and severe lymphocytic atrophy of the thymus. Treated animals also showed an increased severity of atrophy of the bone marrow relative to controls, varying degrees of lymphocytic atrophy of the mandibular and mesenteric lymph nodes and spleen, increased vacuolization and necrosis of cells of the adrenal cortex, focal acinar cell degeneration in the pancreas, and, in males, increased immature sperm forms in the testis and epididymis.

13-Week Studies: 3,3′-Dimethylbenzidine dihydrochloride was administered in drinking water at doses of 300, 500, 1,000, 2,000, and 4,000 ppm. All rats receiving 4,000 ppm and 4/10 males and 1/10 females receiving 2,000 ppm died before the end of the studies. Depressions in final mean body weight relative to controls ranged from 12% to 48% for males and from 9% to 42% for females. Water consumption decreased with increasing dose. At compound concentrations of 300 to 2,000 ppm, mean water consumption was 29% to 83% of control values. Compound-related effects included an increase in the severity of nephropathy relative to controls; hepatocellular necrosis and accumulation of brown pigment (presumably bile) in sinusoidal lining cells; lymphocytic atrophy of the thymus, spleen, and mandibular and mesenteric lymph nodes; atrophy of the bone marrow in the higher-dose groups; degeneration of pancreatic acinar cells; and, in males, immature sperm forms in the testis and epididymis. Decreases in serum triiodothyronine (T_3) values were observed in exposed females, and decreases in mean thyroxin (T_4) concentrations in exposed males and females; no significant changes were observed in thyroid stimulating hormone (TSH) levels in exposed rats.

Based on the decreased survival, reductions in water consumption and body weight gain, and chemical-induced hepatocellular and renal lesions observed in the 13-week studies, the doses selected for the 9- and 14-month drinking water studies of 3,3′-dimethylbenzidine dihydrochloride were 0, 30, 70, and 150 ppm. Seventy rats of each sex were used in the control group, 45 in the low-dose group, 75 in the mid-dose group, and 70 in the high-dose group.

9-Month Studies: Ten rats of each sex in the control and 150 ppm dose groups were evaluated after 9 months. Chemical-related effects observed in exposed animals included alveolar/bronchiolar carcinoma in one male, basal cell carcinoma of the skin in one male, a squamous cell carcinoma of the oral cavity in one female, preputial gland carcinoma in two males, clitoral gland carcinoma in three females, adenocarcinoma of the small intestine in two males, Zymbal's gland carcinoma in two males and three females, hepatocellular carcinoma in two males, and adenomatous polyps of the large intestine in three males. Other effects seen in dosed rats included focal cellular alteration in the liver, lymphoid atrophy in the spleen, and increased severity of nephropathy relative to controls. An increase in serum T_3 values was observed in exposed males, and a decrease in mean T_4 concentrations in exposed males and females. TSH concentrations were increased in exposed male and female rats.

Body Weights and Survival in the 14-Month Studies: The average amount of 3,3′-dimethylbenzidine dihydrochloride consumed per day was approximately 1.8, 4.0, or 11.2 mg/kg for low-, mid-, or high-dose male rats and 3.0, 6.9, or 12.9 mg/kg for low-, mid-, or high-dose female rats. The mean body weight of high-dose males was about 85% of the control value by week 28. By the end of the study, mean body weights of low-, mid-, and high-dose males were 97%, 92%, and 70% of the control values, respectively. Mean body weights of high- and mid-dose females were about 85% of control values at week 32 and week 44, respectively. At the end of the study, mean body weights of exposed females were about 94%, 81%, and 74% of control values for low-, mid-, and high-dose groups, respectively. Because of extensive neoplasia, many exposed males and females were dying or were sacrificed moribund in the first year, and all high-dose males died by week 55. The studies were terminated at weeks 60 to 61, at which time the group survivals were male: control, 60/60; low dose, 41/45; mid dose, 50/75; high dose, 0/60; female: 59/60; 39/45; 32/75; 10/60.

Nonneoplastic Effects in the 14-Month Studies: Increases in nonneoplastic lesions in dosed rats included cystic degeneration and foci of cellular alteration in the liver; exacerbation of nephropathy; and focal or multifocal hyperplasia of the Zymbal's gland, preputial and clitoral glands, and alveolar epithelium.

Neoplastic Effects in the 14-Month Studies: Neoplasms were observed in exposed rats at many sites: skin, Zymbal's gland, preputial and clitoral glands, liver, oral cavity, small and large intestine, mammary gland, lung, brain, and mesothelium. The incidence of these neoplastic effects in male and female rats is summarized in the table at the end of this section.

Genetic Toxicology: 3,3′-Dimethylbenzidine dihydrochloride was mutagenic in *Salmonella typhimurium* strain TA98 with exogenous metabolic activation; it was not mutagenic in strains TA100, TA1535, or TA97 with or without activation. 3,3′-Dimethylbenzidine dihydrochloride induced sister-chromatid exchanges (CHO) and chromosomal aberrations in CHO cells in the absence of exogenous metabolic activation; these effects were not evident in tests with S9 activation. Sex-linked recessive lethal mutations were induced in germ cells of adult male *Drosophilia melanogaster* administered 3,3′-dimethyl-benzidine dihydrochloride in feed or by injection. No reciprocal translocations occurred in *D. melanogaster* germ cells following exposure to 3,3′-dimethylbenzidine dihydrochloride.

Conclusions: Under the conditions of these 14-month drinking water studies, there was *clear evidence of carcinogenic activity** of 3,3′-dimethylbenzidine dihydrochloride for male F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal's gland, preputial gland, liver, oral cavity, small and large intestine, lung, and mesothelium. Increased incidences of neoplasms of the brain may have been related to chemical administration. There was *clear evidence of carcinogenic activity* for female F344/N rats, as indicated by benign and malignant neoplasms of the skin, Zymbal's gland, clitoral gland, liver, oral cavity, small and large intestine, mammary gland, and lung. Increased incidences of neoplasms of the brain and mononuclear cell leukemia may have been related to chemical administration.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 9. A summary of peer review comments and the public discussion on this Technical Report appears on page 11.

Summary of the 14-Month Drinking Water Studies and Genetic Toxicology of 3,3′-Dimethylbenzidine Dihydrochloride

Male F344/N Rats	Female F344/N Rats
Drinking water concentrations	
0, 30, 70, or 150 ppm 3,3′-dimethylbenzidine dihydrochloride	0, 30, 70, or 150 ppm 3,3′-dimethylbenzidine dihydrochloride
Body weights	
Exposed groups lower than controls	Exposed groups lower than controls
2-Year survival rates	
60/60, 41/45, 50/75, 0/60[a]	59/60, 39/45, 32/75, 10/60[a]
Nonneoplastic effects	
Preputial gland: hyperplasia	Clitoral gland: hyperplasia
Liver: cystic degeneration, focal cellular alterations	Liver: cystic degeneration, focal cellular alterations
Lung: hyperplasia	Lung: hyperplasia
Zymbal's gland: hyperplasia	Zymbal's gland: hyperplasia
Neoplastic effects	
Skin basal cell neoplasms: 0/60, 11/45, 54/75, 30/60	Skin basal cell neoplasms: 0/60, 3/45, 10/75, 9/60
Skin sebaceous cell adenoma: 0/60, 0/45, 7/75, 5/60	
Skin keratoacanthomas: 1/60, 1/45, 8/75, 5/60	
Skin squamous cell neoplasms: 0/60, 2/45, 17/75, 27/60	Skin squamous cell neoplasms: 0/60, 3/45, 9/75, 12/60
Zymbal's gland neoplasms: 1/59, 3/45, 32/75, 36/59	Zymbal's gland neoplasms: 0/57, 6/44, 32/73, 42/60
Preputial gland neoplasms: 2/60, 4/45, 6/75, 9/60	Clitoral gland neoplasms: 0/60, 14/45, 42/75, 32/59
Liver neoplasms: 0/60, 0/45, 35/75, 33/60	Liver neoplasms: 0/60, 0/45, 7/74, 4/60
Oral cavity neoplasms: 0/60, 0/45, 4/75, 5/60	Oral cavity neoplasms: 0/60, 3/45, 9/75, 13/60
Small intestine neoplasms: 0/60, 0/45, 4/75, 8/60	Small intestine neoplasms: 0/60, 1/45, 3/75, 5/60
Large intestine neoplasms: 0/60, 0/45, 6/75, 15/60	Large intestine neoplasms: 0/60, 1/45, 7/75, 4/60
	Mammary gland adenocarcinoma: 0/60, 1/45, 3/75, 6/60
Lung neoplasms: 1/60, 0/45, 8/75, 6/60	Lung neoplasms: 1/60, 1/45, 3/74, 4/60
Mesothelioma: 0/60, 0/45, 3/75, 4/60	
	Brain neoplasms: 0/60, 2/45, 2/75, 1/60
Uncertain findings	Mononuclear cell leukemia: 1/60, 3/45, 6/75, 4/60
Brain neoplasms: 0/60, 0/45, 1/75, 2/60	
Level of evidence of carcinogenic activity	
Clear evidence	Clear evidence

Genetic toxicology

Salmonella typhimurium	
Gene mutation:	Positive with S9 in strain TA98; Negative with or without S9 in strains TA100, TA1535, or TA97
Sister chromatid exchanges	
Chinese hamster ovary cells *in vitro*:	Positive without S9
Chromosomal aberrations	
Chinese hamster overy cells *in vitro*:	Positive without S9
Sex-linked recessive lethal mutations	
Drosophila melanogaster in vitro:	Positive administered by injection or in feed
Reciprocal translocations	
Drosophila melanogaster in vitro:	Negative administered in feed

[a] Reduced survival in exposed groups was due to neoplasia.

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence including: animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results (**clear evidence** and **some evidence**); one category for uncertain findings (**equivocal evidence**); one category for no observable effects (**no evidence**); and one category for experiments that because of major flaws cannot be evaluated (**inadequate study**). These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following quintet is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear Evidence** of carcinogenic activity describes studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.

- **Some Evidence** of carcinogenic activity describes studies that are interpreted as showing a chemically related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.

- **Equivocal Evidence** of carcinogenic activity describes studies that are interpreted as showing a marginal increase of neoplasms that may be chemically related.

- **No Evidence** of carcinogenic activity describes studies that are interpreted as showing no chemically related increases in malignant or benign neoplasms.

- **Inadequate Study** of carcinogenic activity describes studies that because of major qualitative or quantitative limitations cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

When a conclusion statement is selected for a particular experiment, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. This should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidences known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or in other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

PEER REVIEW PANEL

The members of the Peer Review Panel who evaluated the draft Technical Report on 3,3′-dimethylbenzidine dihydrochloride on April 25, 1990, are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, Panel members have five major responsibilities:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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SUMMARY OF PEER REVIEW COMMENTS

On April 25, 1990, the draft Technical Report on the toxicology and carcinogenesis studies of 3,3′-dimethylbenzidine dihydrochloride received public review by the National Toxicology Program Board of Scientific Counselors' Technical Reports Review Subcommittee and associated Panel of Experts. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. D. L. Morgan, NIEHS, began the discussion by reviewing the experimental design, results, and proposed conclusions (*clear evidence of carcinogenic activity* for male and female rats). Dr. Morgan explained that the studies were intended to last 24 months but were terminated after 14 months because of rapidly declining survival of exposed animals, due primarily to neoplasia.

Dr. McKnight, a principal reviewer, agreed with the conclusions.

Dr. Zeise, the second principal reviewer, agreed with the conclusions with the exceptions that she felt (1) the marginally increased incidences of benign pheochromocytomas of the adrenal gland medulla in male rats may have been treatment-related, and (2) the marginally increased incidences of mononuclear cell leukemias in female rats may have been treatment-related. Dr. Morgan said pheochromocytomas were commonly occurring tumors in male rats and there was not an increased incidence of hyperplasias. With regard to leukemia, he noted that the study was terminated at 14 months and most leukemias develop after this time. Thus, the rats were not at risk long enough to determine if leukemia was treatment related. Dr. Zeise thought that liver neoplasia in the rat should be reported according to the current classification system, whereby the diagnosis of "neoplastic nodule" is given as either "hepatocellular adenoma" or "hyperplasia." Dr. Morgan explained that "neoplastic nodule" was the accepted terminology when the slides for these liver lesions were read.

Dr. Davis, the third principal reviewer, agreed with the conclusions.

Dr. William Allaben, National Center for Toxicologic Research (NCTR), reported on the 2-year studies of 3,3′-dimethylbenzidine dihydrochloride administrered to BALB/c mice at dose levels ranging from 5 to 140 ppm in drinking water. The only lesions of consequence in these studies were fatal alveolar cell tumors of the lung seen in a dose-related manner in male mice.

Dr. McKnight moved that the Technical Report on 3,3′-dimethylbenzidine dihydrochloride be accepted with the conclusions as written for male and female rats, *clear evidence of carcinogenic activity*. Dr. Davis seconded the motion, which was accepted unanimously with ten votes. Dr. Zeise then moved that mononuclear cell leukemia be added to the conclusion for female rats as "may have been related to chemical administration." Dr. McKnight seconded the motion, which was accepted by nine yes votes to one no vote (Dr. Gold).